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Volatiles associated with different flower stages and leaves of *Acacia cyclops* and their potential role as host attractants for *Dasineura dielsi* (Diptera: Cecidomyiidae)

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Abstract

Acacia cyclops (Fabaceae) is an Australian species which was introduced into South Africa in the nineteenth century. Because of its invasive status in South Africa, a gall midge, *Dasineura dielsi* (Diptera: Cecidomyiidae), was released in 2001 in order to impact its reproduction by inducing galls on the flowers and thereby preventing seed set. Nothing is known about the cues used by *D. dielsi* for locating its host flowers. As part of an initial investigation into whether or not chemical cues might play a role in host finding, we analysed headspace samples of *Acacia cyclops* volatiles from leaves and reproductive parts at different stages (early bud, late bud, early flowering, and senescing flowering stages) using gas chromatography–mass spectrometry (GC–MS). In total, 72 different compounds were detected of which 62 were identified. The analyses showed that open flowers, the stage used by *D. dielsi* for oviposition, and yellow buds had similar odour compositions with (*Z*)-3-hexen-1-ol acetate, 4-oxoisophorone, (*Z*)-β-ocimene, an unknown aliphatic compound, heptadecane, and nonadecane dominating in open flowers. Leaf volatiles were distinct from those in the reproductive plant parts by their high relative amount of (*Z*)-β-ocimene. (*Z*)-3-Hexen-1-ol acetate had its maximum relative amount in the green bud samples and was much lower in the later floral stages. In contrast, 4-oxoisophorone peaked in yellow buds and open flowers with little or none of it found in younger or older stages. The volatile compounds of the different flower stages and leaves are discussed in relation to their potential role as attractants used by the biocontrol agent *D. dielsi* to locate its host plant.

Keywords: *Acacia cyclops*; Biological control; *Dasineura dielsi*; Headspace analysis; Spatial odour patterns; 4-Oxoisophorone

1. Introduction

Biological control of weeds is an important discipline of classical biological control and is practiced in many countries (Palmer et al., 2010; Timmons, 2005; Zimmermann et al., 2004) to protect agricultural crops and conservation of biodiversity (Zimmermann et al., 2004). It is a practice in which the alien invasive plants' natural enemies e.g. host-specific plant-feeding insects, mites and pathogens are transferred from their country of origin and after rigorous host-specificity tests are released into the country where the plants have become a problem (Zimmermann et al., 2004). Host-specificity tests ensure that the introduced biocontrol agent can maintain its populations in only one, or a limited number of closely related species of host plants (Zimmermann et al., 2004). Biologically relevant testing methods are based on investigations of the physiological, morphological, phenological, entomological, and chemical bases of host restriction (McEvoy, 1996). Host chemistry however has not often been used in determining the host range of a biocontrol agent, and therefore the suitability of an insect as a biocontrol agent (Jordon-Thaden and Louda, 2003). After an investigation into the chemistry of thistles to determine why a biocontrol agent expanded its host range from exotic thistles to native thistles, Jordon-Thaden and Louda (2003) suggested that consideration of contemporary chemical profiles to supplement host-specificity tests based on phylogenetic relationships will contribute to a clearer prediction of potential host plant use and ecological impacts.

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Acacia cyclops A. Cunn. ex G. Don (Fabaceae), originating in south-western Australia, became invasive after it was introduced into South Africa in the early 1800s for dune stabilisation (Adair, 2005; Impson et al., 2004; Moseley et al., 2009). Reproduction is entirely sexual and relies on the production of copious quantities of seeds which accumulate as large seed banks and which remain viable for many years (Richardson and Kluge, 2008). Today it has invader status (category 2) in South Africa as it impacts negatively on the biodiversity of the environment (Henderson, 2001). It also grows in and reduces water production in catchment areas, amongst other impacts (Richardson and Kluge, 2008). Contrary to the many negative ecological aspects, it also has economic benefits as it is an excellent source of firewood, and as such it generates considerable income for lower income communities in South Africa (Dennill and Donnelly, 1991; Impson et al., 2004). For this reason, a compromise was reached by releasing biological control agents that reduce seed set without affecting vegetative growth and, to date, two complementary biocontrol agents have been released in South Africa: a weevil, *Melanterius servulus* (Coleoptera: Curculionidae) which feeds directly on seeds (Dennill and Donnelly, 1991; Impson et al., 2004) and a midge, *Dasineura dielsi* Rübsaamen (Diptera: Cecidomyiidae), which induces galls on the flowers and thereby prevents seed set (Adair, 2005).

Dasineura dielsi, which also originated from Australia (Kolesik et al., 2005) is short lived as an adult (approximately 2 days) with its total life cycle, from egg stage through three larval stages and a pupal stage, taking place in the gall (Adair, 2003, 2005; Kolesik et al., 2005). Soon after emergence from the gall, mating takes place and the female starts searching for oviposition sites, depositing her eggs on the perianth tube of the ovaries of open *A. cyclops* flower heads (Adair, 2003, 2005). Gall formation commences with larval feeding. Inflorescences of *A. cyclops* consist of a number of flowers. Each flower in which eggs have been laid develops into a multi-chambered gall and the entire inflorescence forms a spherical cluster of protruding, convoluted and elongated galls (Adair, 2003, 2005).

Acacia cyclops is the primary host of *D. dielsi* (Adair, 2003, 2005). The extended flowering period of *A. cyclops* sustains the multivoltine life strategy of *D. dielsi* which has up to five generations per annum (Adair, 2003, 2005; Henderson, 2001). Other Australian *Acacia* species invasive in South Africa, such as *A. melanoxylon*, *A. longifolia* and *A. saligna*, are occasionally used as hosts by *D. dielsi* (Post et al., 2010). Considering that the midges are weak flyers (Kolesik, 2000), and must find a suitable host within the short period of their ephemeral life span, they need clear cues to indicate the presence of the correct host plant species with suitable flowers. The infloresences of *A. cyclops* are bright yellow and large enough to be visually apparent, they are largely visually-indistinguishable from many other co-occurring *Acacia* species and are generally obscure among the foliage of the plants on which they are borne. These features emphasize that olfactory cues are also likely to play a central role in this plant–insect interaction but nothing is known about the olfactory responses of *D. dielsi*.

At the same time, little is known about the volatile chemistry of most species of *Acacia* (Seigler, 2003) whose comprehensive report on the phytochemistry of *Acacia*, only mentions plant volatiles from *A. farnesiana*. Floral volatiles have been described from *A. farnesiana*, *A. berlandieri*, and *A. rigidula* (all three native to North America) (Flath et al., 1983), *A. praecox*, *A. caven*, *A. aroma* (all three from Argentina) (Lamarque et al., 1998; Zygadlo et al., 1996), and from *A. karroo*, an African species (Kaiser, 1997).

Many earlier studies of plant volatiles, such as the one by Flath et al. (1983), used a vacuum steam distillation, hydrodistillation or solvent extraction method to extract the volatiles. All these techniques however remove substances from the plant tissue itself, including compounds that may not be emitted by the plant into the surrounding air. Furthermore, such methods may dilute, or entirely miss, emitted volatiles, especially those that are synthesized continuously. More reliable information on the occurrence of naturally emitted blends of volatiles can be obtained using dynamic headspace sampling, whereby volatiles are trapped on a polymer and then extracted with solvent or thermally desorbed. The latter is a highly sensitive method that was used in the present investigation to characterize volatile emissions from leaves, green buds, yellow buds, open flowers and senescing flowers of *A. cyclops* in order to identify potential chemical cues that could be used by *D. dielsi* to locate flowers.

2. Methods

2.1. Collection of volatile samples from different flowering stages and leaves

From the perspective of the objectives of this study, in-situ headspace collection of *A. cyclops* flower volatiles was not feasible due to the way the inflorescences are borne on the stems. The globose flower heads occur sporadically, and are individually interspersed among the leaves. Preliminary headspace samples inadvertently included scent from leaves and a whole range of flower stages with the flower scent concentration after GC–MS analysis of the samples too weak to confidently compare the scent compositions to those of the other flower stages. Therefore, despite the risk of obtaining high levels of green leaf volatiles characteristic of wounded foliage (Arimura et al., 2001; Grison et al., 1999), it was decided to pick the flowers, buds and leaves separately for headspace volatile collection.

Plant cuttings containing leaves, and buds and flowers of different stages were collected from 25 “impartially” selected *A. cyclops* trees at the Koeberg Nature Reserve (S 33° 39′ 14.8″ E 18° 26′ 00.4″) near Melkbosstrand in the Western Cape, South Africa. The plant cuttings were transported to a laboratory where on arrival the required plant parts (leaves, green buds, yellow buds, open flowers and senescing flowers) were carefully removed from the cuttings with a pair of forceps. Buds and flowers thus picked out were pooled to create five samples of the leaves and five samples of each of the selected flower stages (green buds, yellow buds, open flowers and senescing flowers), thus giving five replicates of each of the required plant parts.

Each sample contained material from five individual trees. The leaves, buds and flowers removed from the plant cuttings were enclosed within a polyacetate oven bag (25 cm × 30 cm; Kalle Bratschlauch, Wiesbaden, Germany) for dynamic headspace collection as described by Dötterl et al. (2005a). Material for all samples except green buds was collected on 21 January 2010. The green bud samples were collected on 26 March 2010.

Volatiles were trapped in an adsorbent tube by using a membrane pump (Spectrex PAS-500, Redwood City, California, USA) with a flow rate of 100 ml/min for 30 min. Air was drawn from the bag through the adsorbent tube which was filled with a mixture of 1.5 mg Tenax-TA® (mesh 60–80, Supelco, Bellefonte, USA) and 1.5 mg Carbotrap® (mesh 20–40, Supelco, Bellefonte, USA). To distinguish between plant volatiles and ambient contaminants, after each replicate of samples (leaf and each of the selected flower stages), surrounding air was collected separately as control samples for comparison purposes. After taking the headspace samples the buds and flowers in the sample bags were counted.

2.2. GC–MS analyses

GC–MS analysis of floral scent samples was carried out using a Varian CP-3800 GC (Varian, Palo Alto, California) with a 30 m × 0.25 mm internal diameter (film thickness 0.25 μm) Alltech EC-WAX column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode at 70 eV (Shuttleworth and Johnson, 2010). Cartridges were placed in a Varian 1079 injector equipped with a 'Chromatoprobe' thermal desorption device (Amirav and Dagan, 1997). The flow of helium carrier gas was 1 ml/min. The injector was held at 40 °C for 2 min with a 20:1 split and then increased to 200 °C at 200 °C/min in split-less mode for thermal desorption. The temperature of the GC oven was held for 3 min at 40 °C, where after it was ramped up to 240 °C at 10 °C/min and held there for 12 min. Identification of compounds included the use of the Varian Workstation software with the NIST 05 mass spectral library (NIST/EPA/NIH Mass Spectral Library (data version: NIST 05; MS search software version 2.0 d)) as well as verification by using retention times of authentic standards and published Kovats indices (references in the NIST 05 library) (El-Sayed, 2009) wherever possible. Compounds present at similar abundance in the control samples were considered to be contaminants and excluded from analysis. The emission rates per hour per flower were quantified by injecting known amounts of methyl benzoate into thermo-desorption cartridges that were then thermally desorbed using the same methods that were applied to the biological samples.

2.3. Statistical analysis

All statistical analyses employed the statistical package PRIMER v6 (Clarke and Gorley, 2006; Clarke and Warwick, 2001). Since the total amount of volatiles emitted varied greatly among individual samples, differences in scent composition of each sample were assessed using percentages of individual compounds in the bouquet (relative amounts derived from total peak areas). All compounds from the samples taken for leaves, green and yellow buds, and open and senescing flowers were identified. To arrive at the relative amounts of compounds for leaves, green and yellow buds, and open and senescing flowers, the average relative amounts per compound were calculated and then square-root transformed. The significance level of differences in scent profiles of different plant parts was assessed with an analysis of similarities (ANOSIM) (Clarke and Gorley, 2006) with 10,000 random permutations. The extent to which individual compounds contributed to the overall dissimilarity among the plant parts was subsequently assessed with the SIMPER procedure (factor: plant parts) (Clarke and Warwick, 2001). Non-metric multidimensional scaling was used (based on the Bray–Curtis similarities) in PRIMER v6 to ordinate the scent samples of the different plant parts in order to visualize similarities among the individual samples. To analyse whether the compounds in the scent showed an even occurrence between the different floral stages and leaves the evenness ($E = H'/\ln C$; where H' is the Shannon index (Shannon and Weaver, 1949) and C is the total number of compounds) was calculated (Stiling, 1999). The evenness factor is constrained between 0 and 1 (Stiling, 1999); the higher the factor the more even the occurrence of the compounds. To determine if there were significant differences in the mean rates of volatile emission from various flowers stages, we used one-way ANOVA, followed by the Tukey multiple range test. To improve normality and homoscedasticity of the data, they were log-transformed prior to this analysis.

3. Results

The chemical composition of leaves and various floral stages, green buds, yellow buds, open flowers, and senescing flowers of *Acacia cyclops* is summarized in Table 1. In total 72 compounds were found in the scent emitted by the leaves and different floral stages of *A. cyclops*. They belong to six different chemical classes, namely aliphatic compounds, benzenoids, monoterpenoids, sesquiterpenoids, irregular terpenes, and nitrogen containing compounds. Most of the compounds were from the aliphatic (27) and monoterpene (17) classes. Twenty of the 72 compounds were unique or occurring in only one sample type; their total relative abundance however was low, never exceeding 5% (Table 1). Qualitative differences in the scent composition between leaves and different flower stages are also evidenced by the number of compounds found. Of the 72 compounds found in all samples, 44 were present in leaf samples, 28 compounds were emitted by samples from green buds, 43 compounds were detected in the yellow bud samples, the open flower samples emitted 44 compounds and the samples from senescing flowers contained 44 volatiles.

Only 13 out of 72 compounds occurred in all five sample types. Nine of these 13 compounds showed a maximum relative amount of less than 5% and for all nine the maximum relative amounts were found in the senescing flowers. The four remaining compounds common to all sample types were (*Z*)-3-hexen-1-ol acetate, 4-oxoisophorone, an unknown aliphatic compound (KRI = 1697), and (*Z*)-β-ocimene, showed large

Table 1
Relative amounts (%) of compounds identified by GC–MS from headspace samples of different flower stages (green buds, yellow buds, open flowers, and senescing flowers) and leaves of *Acacia cyclops* (number of occurrences in the five samples per sample type in brackets). Scent compounds are listed according to compound class and Kovats retention index (KRI). CAS = Chemical Abstracts Service Registry Number. tr = trace amount (< 0.1 % of total sample). Compound identification criteria: a = comparison of MS with published data (e.g. NIST library); b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard. Unknowns that did not reach at least 1% of relative amount in any sample were pooled with the superscript digit indicating the number of pooled compounds. Mass fragments for unknowns are listed with the base peak first, followed by the other fragments in order of decreasing abundance.

Component	KRI	CAS	Leaves	Green buds	Yellow buds	Open flowers	Senescing flowers
Number of compounds			44	28	43	44	45
Aliphatic compounds							
Aliphatic aldehydes							
(*E*)-2-Hexenal[b]	1242	6728-26-3	–	0.8 (3)	tr	0.5 (2)	1 (3)
(*E*,*Z*)-2,6-Nonadienal[b]	1608	557-48-2	–	–	tr	–	0.7 (3)
Aliphatic esters							
Hexyl acetate[b]	1288	142-92-7	–	1.1 (2)	2.1 (5)	3.1 (5)	4.3 (5)
(*E*)-2-Methyl hexenoate[b]	1308	13894-63-8	0.4 (5)	–	–	–	–
(*Z*)-3-Hexenyl acetate[b]	1333	3681-71-8	9.3 (5)	45 (5)	21.3 (5)	24.1 (5)	22.8 (5)
(*E*)-2-Hexenyl acetate[b]	1348	2497-18-9	0.2 (1)	0.6 (2)	tr	–	–
(*E*)-3-Hexenyl butyrate[b]	1474	53398-84-8	0.1 (5)	–	–	–	–
(*Z*)-3-Hexenyl hexanoate[b]	1704	31501-11-8	tr	–	–	–	–
Unidentified aliphatic esters			tr[1]	0.4[2]	0.1[1]	tr[1]	–
Aliphatic alcohols							
1-Hexanol[c]	1352	111-27-3	–	–	0.2 (2)	0.3 (2)	5.9 (5)
(*Z*)-3-Hexen-1-ol[b]	1395	928-96-1	1.6 (4)	2.1 (5)	1.1 (3)	0.6 (2)	2.5 (3)
(*E*)-2-Hexen-1-ol[b]	1396	928-95-0	–	–	0.3 (1)	0.1 (1)	1.3 (1)
(*Z*)-2-Hexen-1-ol[b]	1414	928-94-9	tr	0.6 (5)	–	–	–
4-Methyl-1-heptanol[a]	1529	817-91-4	–	–	–	–	0.2 (1)
6-Methyl-1-heptanol[a]	1531	1653-40-3	–	–	–	–	0.8 (3)
2,3-Butanediol[b]	1587	513-85-9	–	–	–	–	0.2 (1)
Aliphatic alkanes							
Hexadecane[c]	1598	544-76-3	0.3 (4)	–	0.5 (2)	0.3 (2)	0.5 (2)
Heptadecane[c]	1701	629-78-7	0.4 (5)	–	13.2 (5)	8.3 (3)	2.5 (4)
Octadecane[c]	1801	593-45-3	–	–	0.2 (4)	0.2 (4)	tr
Nonadecane[c]	1903	629-92-5	0.2 (1)	–	3.2 (3)	5.7 (4)	1.6 (2)
Eicosane[c]	2001	112-95-8	–	–	tr	tr	1.1 (1)
Heneicosane[c]	2099	629-94-7	–	–	tr	0.3 (5)	0.3 (3)
Tricosane[c]	2293	638-67-5	–	–	tr	0.2 (5)	0.6 (5)
Unidentified aliphatic compound m/z: 56,85,125,43,41,69,153,55,83,39	1697		0.1 (4)	tr	6.3 (5)	11.3 (5)	1.5 (5)
Benzenoids							
Benzaldehyde[c]	1551	100-52-7	0.4 (5)	0.6 (5)	0.7 (5)	0.5 (5)	1.9 (5)
Methylbenzoate[c]	1650	93-58-3	0.2 (5)	0.4 (5)	0.2 (5)	–	–
Benzyl acetate[c]	1755	140-11-4	–	0.1 (5)	tr	–	–
Methyl salicylate[c]	1806	119-36-8	2.7 (5)	0.7 (5)	0.6 (4)	–	0.2 (3)
Benzyl alcohol[c]	1902	100-51-6	0.4 (4)	0.4 (5)	0.7 (2)	0.2 (1)	4.1 (4)
Phenylethyl alcohol[c]	1938	60-12-8	0.2 (5)	0.2 (5)	0.2 (5)	tr	0.7 (5)
Dimethyl salicylate[b]	2093	606-45-1	tr	–	tr	tr	tr
Benzyl tiglate[a]	2139	37526-88-8	–	–	tr	tr	0.4 (4)
Unidentified benzonoids	2135		–	–	–	tr[1]	–
Monoterpenoids							
3-Carene[b]	1122	13466-78-9	0.8 (2)	–	–	–	–
α-Pinene[c]	1087	80-56-8	–	–	–	–	2.9 (2)
β-Pinene[c]	1199	127-91-3	0.2 (3)	–	–	–	–
Limonene[c]	1225	138-86-3	1.2 (5)	0.1 (4)	1.8 (5)	1.6 (5)	3.2 (5)
(*Z*)-β-Ocimene[b]	1252	3338-55-4	70.7 (5)	44.9 (5)	19.3 (5)	10.6 (5)	18.2 (5)
(*E*)-β-Ocimene[b]	1271	3779-61-1	0.1 (2)	–	–	–	–
(*E*)-Linaloloxide (furanoid)[c]	1457	34995-77-2	0.8 (5)	–	0.6 (5)	0.7 (5)	2.5 (5)
(*E*,*E*)-2,6-Dimethyl-1,3,5,7-octatetraene[a]	1466	460-01-5	1.3 (5)	tr	–	tr	–
(*Z*)-Linaloloxide (furanoid)[c]	1486	5989-33-3	–	–	0.2 (3)	tr	–
β-Linalool[c]	1556	78-70-6	0.9 (5)	0.1 (5)	0.4 (4)	0.3 (5)	1.1 (5)

Table 1 (*continued*)

Component	KRI	CAS	Leaves	Green buds	Yellow buds	Open flowers	Senescing flowers
Number of compounds			44	28	43	44	45
Monoterpenoids							
β-Citronellal[b]	1609	432-25-7	–	–	–	–	0.1 (1)
Isobornyl acetate[a]	1610	125-12-2	tr	–	tr	0.1 (5)	tr
α-Terpineol[c]	1716	10482-56-1	0.1 (4)	tr	–	0.2 (5)	0.3 (4)
Borneol[a]	1725	507-70-0	tr	tr	–	–	–
6,6-Dimethyl-2-methylene-Bicyclo[3.1.1]heptan-3-ol[a]	1731	5947-36-4	tr	–	–	–	–
D-Verbenone[a]	1740	18309-32-5	tr	–	–	tr	0.2 (5)
Epoxylinalol[a]	1758	14049-11-7	0.4 (5)	–	0.4 (5)	0.3 (5)	1 (4)
(*Z*,*Z*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1824	141119	tr	–	–	–	tr
(*E*,*E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1836	141118	0.8 (5)	tr	0.2 (4)	tr	–
Unidentified monoterpenoids			tr[1]	–	–	–	tr[1]
Sesquiterpenoids							
α-Bulnesene[a]	1599	3691-11-0	–	–	–	0.4 (3)	–
β-Caryophyllene[c]	1625	87-44-5	0.4 (5)	0.2 (5)	0.4 (5)	0.8 (5)	3.7 (5)
α-Farnesene[b]	1765	502-61-4	2.3 (5)	tr	0.7 (5)	0.5 (5)	2.4 (4)
Caryophyllene oxide[b]	2021	1139-30-6	0.1 (1)	–	tr	0.2 (5)	0.3 (4)
Unidentified sesquiterpenoids			tr	–	0.2	0.3	1.1
Irregular terpenes							
6-Methyl-5-hepten-2-one[b]	1354	110-93-0	2 (5)	0.6 (5)	3.4 (5)	1.7 (3)	4.4 (4)
2,6-Dimethyl-6-octanol[a]	1437	78-69-3	tr	tr	–	–	–
2,6-Dimethyl-7-octen-2-ol[a]	1474	18479-58-8	–	–	–	tr	–
2,7-Dimethyl-2,6-octadien-1-ol[a]	1522	16736-42-8	–	–	–	tr	–
Unidentified irregular terpene m/z: 138,96,68,67,95,39,41,42,40,69	1535		–	–	0.3 (1)	1.4 (4)	–
4-Methoxy-2,2,6-trimethyl-cyclohexanone[a]	1630	17429-03-7	–	–	tr	0.2 (4)	–
4-Oxoisophorone[a]	1722	1125-21-9	0.2 (4)	tr	20.2 (5)	23.7 (5)	2.9 (5)
2,2,6-Trimethyl-1,4-cyclohexanedione[a]	1808	20547-99-3	–	–	0.4 (5)	0.7 (5)	0.1 (1)
Nitrogen containing compound							
Unidentified nitrogen containing compound[a] m/z:73,56,59,41,86, 55,39,69,72,57	1507		0.3 (5)	0.4 (1)	–	–	–
Indole[c]	2468	120-72-9	0.5 (5)	–	tr	–	tr

quantitative differences in relative amounts: (*Z*)-β-ocimene accounted for 70.7% of the plant volatiles emitted by the leaves followed by 9.3% of (*Z*)-3-hexen-1-ol acetate, whereas in green buds (*Z*)-3-hexen-1-ol acetate and (*Z*)-β-ocimene together dominated the compound profile with approximately 45% each. In yellow buds, the same two compounds plus 4-oxoisophorone dominated with about 20% each, followed by heptadecane (13.2%) and an unknown aliphatic compound (KRI=1697) (6.4%). The scent of open flowers was dominated by (*Z*)-3-hexen-1-ol acetate (24.1%), 4-oxoisophorone (23.7%), (*Z*)-β-ocimene (10.6%), followed by the unknown aliphatic compound (KRI=1697) (11.3%), heptadecane (8.3%), and nonadecane (5.7%). In senescing flowers, two of the common compounds, (*Z*)-3-hexen-1-ol acetate (22.8%) and (*Z*)-β-ocimene (18.2%) still accounted for 41% of the total plant volatiles emitted, followed by 1-hexanol (5.9%), and many other compounds with <5%. Thus, in senescing flowers, apart from the pattern that holds across all the sample types with respect to (*Z*)-3-hexen-1-ol acetate and (*Z*)-β-ocimene, the compound occurrence is spread more evenly across the compound profile. The evenness of the compound occurrence in the senescing flowers chemical profile was 0.75; that of leaves and green buds both 0.37, and for yellow buds and open flowers both 0.61.

(*Z*)-3-Hexen-1-ol acetate had its maximum relative amount (45%) in the green bud samples; it decreased in the yellow floral stages, though there was also a high relative amount for each of the three yellow stages (from 21 to 24%). 1-Hexanol was present in the yellow floral stages initially in very small relative amounts (0.23–0.34%) and then reached a maximum (5.9%) in the senescing flowers stage. 4-Oxoisophorone and the unknown aliphatic compound (KRI=1697) followed the same patterns. Both compounds were present in all sample types, very small in the green stages, peaked in the open flower stage (23.7% and 11.3%) with a comparative relative amount in the yellow buds stage, and dropped off drastically in the senescing flower stage.

Together with other alkanes, nonadecane and especially heptadecane are prominent in the samples analysed. The relative amount of heptadecane had its maximum (13.2%) in the yellow buds floral stage and nonadecane (5.7%) in the open flowers stage. (*Z*)-β-ocimene was present in a very high relative amount in the leaves (70.7%), and decreased in abundance through the two bud stages, with a minimum in the open flower stage (10.6%) and then increased again in the senescing flowers stage to almost the same level as in the yellow buds.

In summary, there were marked differences between the flower stages and the leaves based on compound classes (Fig. 1). For example, all flower material emitted close to 50% aliphatic

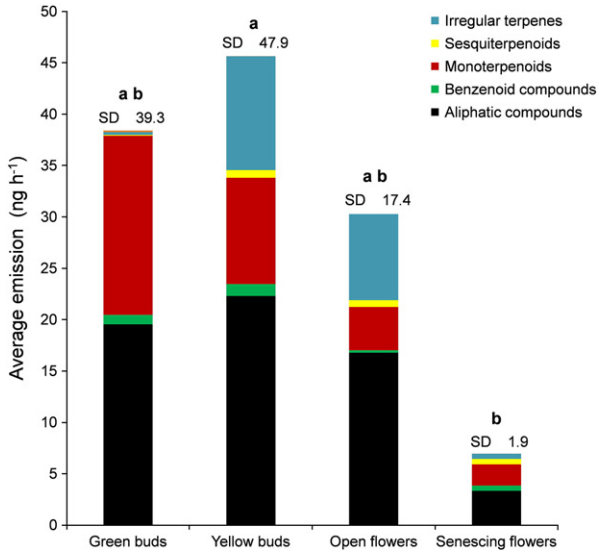

Fig. 1. Absolute amounts of the volatile emissions (ng/h) from different bud and flower stages of *Acacia cyclops*. Stages having the same letters are not significantly different at the 5% level (Tukey HSD, $F_{3,19}=4.024$; $p=0.026$); $n=5$.

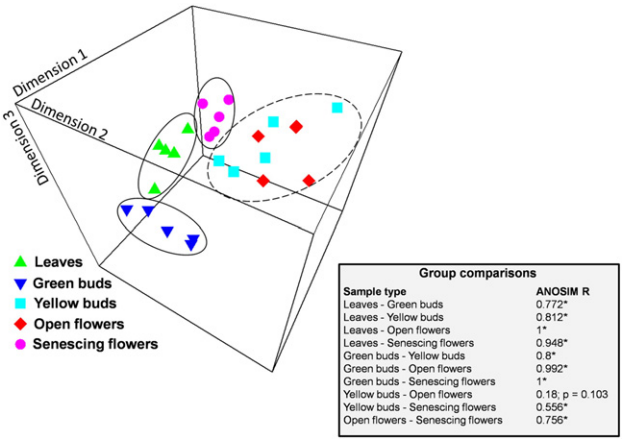

Fig. 2. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarities of the odour composition (72 compounds) of different flower stages and leaves of *Acacia cyclops*. 3D-NMDS stress value=0.06; ANOSIM Global $R=0.784$, $p<0.001$. Group comparisons (ANOSIM) are shown in the box. With the exception of yellow buds vs open flowers ($R=0.18$; $p=0.103$ all comparisons) all group comparisons were significant at the 1% level.

compounds, and leaves and green buds showed higher relative amounts of monoterpenes compared to the other plant parts, whereas yellow buds and open flowers had the highest relative amount of irregular terpenes, and senescing flowers had the highest relative amounts of sesquiterpenes and benzenoids. Closer inspection revealed that these summarized compound class differences were mainly due to single dominating compounds (Table 1): (1) leaves were distinct from reproductive plant parts, especially open flowers, by their high relative amount of (*Z*)-β-ocimene; (2) with respect to reproductive units, (*Z*)-3-hexen-1-ol acetate had its maximum relative amount in the green bud samples and was much lower in the following floral stages; (3) in contrast, 4-oxoisophorone peaked in yellow buds and open flowers with little or none of it found in younger or older stages, and an unknown aliphatic compound (KRI=1697) and hepta- and nonadecane exhibited a similar trend albeit on a much lower level, with the unknown and nonadecane peaking in open flowers, and heptadecane in mature yellow buds.

The senescing flower stage was characterized by a drop in volatile emission to a mean 7 ng/h per flower (Table 1). This contrasts sharply with the more than 30 ng/h per flower of the buds and open flower stages. The yellow buds and senescing flower stages differed significantly in emission rates (Fig. 1).

The overall separation of the plant volatile profiles, based on Bray–Curtis similarities of the different plant parts was highly significant (Fig. 2; 3D-NMDS stress value=0.06; ANOSIM $R=0.784$, $p<0.001$). Pair-wise comparison revealed highly significant differences between leaves and the four floral stages (range of R-values is from 0.768 to 1), between green buds and the three yellow floral stages (range of R-values is from 0.796 to 1) and between open flowers and senescing flowers (R-value=0.756). The pair-wise comparison however revealed only slight differences between yellow buds and open flowers (R-value=0.18; $p=0.103$) and a moderate difference between yellow buds and senescing flowers (R-value=0.556; $p=0.08$). The dissimilarity indices calculated by the SIMPER method revealed a dissimilarity between the green stages (leaves and green buds) of 45.55% (meaning a similarity of >50%). The main compounds contributing to this dissimilarity were (*Z*)-3-hexen-1-ol acetate (13.65%) and (*Z*)-β-ocimene (10.69%). The dissimilarity indices calculated for the yellow flower stages (yellow buds, open flowers and senescing flowers) ranged from 35.39% to 45.89%. The main compounds contributing to these dissimilarities were (*Z*)-3-hexen-1-ol acetate, the unknown aliphatic compound (KRI 1697), heptadecane, 4-oxoisophorone and nonadecane. (*Z*)-β-ocimene differentiated the senescing flower stage from the yellow buds and open flower stages; and 1-hexanol differentiated the senescing flowers stages from the yellow buds and open flowers. Thus, in all three cases there were similarities of >50%, with the largest similarity between yellow buds and open flowers. The calculated dissimilarity indices between green leaves and green buds and the three floral yellow stages ranged from 51.06% to 62.88%. 4-Oxoisophorone and (*Z*)-β-ocimene contributed strongly to the dissimilarity of all six pairs between the green leaves and buds and yellow floral material. Heptadecane and (*Z*)-3-hexen-1-ol acetate contributed to the dissimilarity in all cases except the leaves-senescing flowers pair and the leaves-open flowers pair respectively.

4. Discussion

4.1. Scent variation of different floral parts and leaves

Variability in scent compositions due to time and spatial patterns are common, e.g. variability in the floral scent composition of different floral parts has been reported for *Silene latifolia* (Dötterl and Jürgens, 2005); variability in scent emissions from pollen, leaves and floral parts from garland a popular food plant from China (Flamini et al., 2003); temporal

variability expressed in the different stages of ripeness (green, ripe and dried) of fruit of Japanese Pepper (Jiang and Kubota, 2004). In line with this commonality, differences in the scent composition of the different floral stages and leaves of *A. cyclops* (Figs. 1 and 2) were also found.

The very small relative amounts of compounds that were unique to each of the four flower phenophases or leaves, suggests that differences in the volatile emissions were mainly quantitative. Although distinct in their odour composition, leaves and green buds of *A. cyclops* with 44 and 28 compounds, respectively, were more similar to each other than they were to the other three floral stages which each emitted more than 40 volatiles (Table 1; Fig. 2). The lowest separation was found between yellow buds and open flowers. Senescing flowers were characterized by more evenness in compound distribution and lacked the dominance of certain compounds e.g. (*Z*)-β-ocimene from the leaves and hepta- and nonadecane, the unknown aliphatic compound and 4-oxoisophorone from the other floral stages. Biologically there is no particular function to be ascribed to senescing flowers, and the lack of dominant components seems to be congruent with the position of senescing flowers in the life cycle of flowers. This may also explain the higher relative amounts of sesquiterpenes and benzonoids observed in the senescing flowers (Fig. 1; Table 1).

(*Z*)-3-Hexen-1-ol acetate, a well-known green leaf volatile (GLV), has been reported for many plant genera (Knudsen et al., 2006). Although it commonly occurs in intact and healthy plant tissues (Matsui, 2006); it increases rapidly as a product of the lipoxygenase pathway when tissues are wounded and is often characteristic of wounded foliage (Arimura et al., 2001; Grison, et al., 1999). It is therefore possible that the high levels of this compound may be due to the cutting of the plant material and the "picking" of the buds and flowers from the plant stems. However, the relative and absolute levels of (*Z*)-3-hexen-1-ol acetate decrease as the inflorescences of *A. cyclops* mature. This suggests that the presence of (*Z*)-3-hexen-1-ol acetate is not just due to the sampling method used, but also indicate presence under intact circumstances. Decreasing levels of (*Z*)-3-hexen-1-ol acetate as inflorescences mature has also been reported for a cultivar of raspberry, *Rubus idaeus* (Robertson et al., 1995).

(*Z*)-β-ocimene, a common floral volatile (Knudsen et al., 2006) was one of the dominant compounds of the scent of *A. cyclops* and has also been found in *Acacia farnesiana* (Flath et al., 1983). As what happened in this study, levels of most volatiles associated with GLVs decrease and levels of monoterpenes such as limonene increase as inflorescences mature in red raspberry (Robertson et al., 1995). The very high level of (*Z*)-β-ocimene, particularly in the green leaves, may be a compounded effect of several influences of which the method of preparing the plant material for sampling may also have contributed. A seasonal effect (the green bud samples were collected in March which is the start of the South African autumn season whereas the other samples were collected in January which is in the middle of the South African summer) may explain why green buds also had relatively high amounts of (*Z*)-β-ocimene.

Even though the relative amount of 1-hexanol in the volatile profile of the senescing flowers was low, it was still 26-fold and 17-fold more than that emitted from the yellow buds and open flowers respectively. Schade et al. (2001) also measured an increase in 1-hexanol as flower developing stages progressed from young buds to senescing flowers.

4-Oxoisophorone is not a common floral compound (Knudsen et al, 2006) and to our knowledge it has not been described in other *Acacia* species so far. In our study 4-oxoisophorone was the highest in yellow stages of both enlarged buds and open flowers. 4-Oxoisophorone is a flavour compound of carotenoid origin (Goff and Klee, 2006). In plants, the presence of carotenoids is revealed by the rich colour of flowers, fruits and storage organs in the yellow-to-red part of the visual spectrum (Farré et al., 2010). Carotenoids accumulate in different plant tissues and help to attract pollinators to flowers and aid in seed dispersion through the strong red, orange and yellow colours they impart and which attract frugivores to ripe fruits (Goff and Klee, 2006; Simkin et al., 2010).

The seven alkanes that have been identified in the odour of *A. cyclops* contributed a substantial amount to the total volatile emissions of the yellow buds and the open flowers. The scent emission rates of buds and flowers which increased substantially as the buds and flowers developed and matured and declined again as the flowers senesced reflected a pattern which has also been observed in other flowers (e.g. Dötterl et al., 2005a; Verdonk et al., 2003).

It is possible that these significant differences in the relative amounts of compounds in the different stages of the plant's flowering cycle, particularly of the dominant compounds [(*Z*)-3-hexen-1-ol acetate; 4-oxoisophorone; the unknown aliphatic compound (KRI = 1697); (*Z*)-β-ocimene; and several aliphatic alkanes] might play a role in the interactions between *A. cyclops* and its associated insect fauna.

4.2. Volatiles as potential host finding signals for D. dielsi

In common with most herbivorous insects, at least two species of gall midges e.g. *Dasineura mali*, an apple leafcurling midge, and *Sitodiplosis mosellana*, the orange wheat blossom midge, have been shown to respond to plant volatiles while orientating towards their hosts (Birkett et al., 2004; Galanihe and Harris, 1997). A longstanding debate exists whether either species-specific compounds or specific ratios of ubiquitous compounds are utilised in host finding by phytophagous insects. Support exists for both points of view but with greater support for the latter (Bruce et al., 2005; Schoonhoven et al., 2005). The n-alkanes in particular heptadecane and (*Z*)-β-ocimene found in the open flower samples of *A. cyclops* are found in many plant volatile bouquets and 4-oxoisophorone is known to attract some Lepidoptera and Hymenoptera.

Dasineura dielsi only oviposits on the open yellow flowers of *A. cyclops* (Adair, 2003). The scent of the open flowers of *A. cyclops* is characterized by high levels of 4-oxoisophorone, the unknown aliphatic compound and the n-alkanes heptadecane and nonadecane. Whilst no information currently exists about the role of the unknown aliphatic compound, both 4-oxoisophorone and n-alkanes are known as attractants to insects. n-Alkanes emitted from corn (*Zea mays*) have been shown to stimulate oviposition (Krokos et al., 2002), or contribute to stimulation of oviposition

(Derksen, 2006), on the plants. 4-Oxoisophorone is a well-known moth attractant (Guédot et al., 2008) and is also known to stimulate antennal responses of butterflies (Andersson, 2003) and bees (Dötterl et al., 2005). Bees are common pollinators of many *Acacia* species (Stone et al., 2003) and butterflies have been observed as pollinators of *Acacia caesia* in India (Raju et al., 2006). Investigations are ongoing to determine the role of these compounds in host finding by *D. dielsi*.

While the yellow buds stage also contains high levels of both 4-oxoisophorone and the unknown aliphatic compound, from a morphological perspective the midge cannot oviposit in the buds due to the compact structure of the flower bud and the non-sclerotised ovipositor of *D. dielsi* (Kolesik et al., 2005). Hence the role of these compounds in this floral stage is still unclear. (*Z*)-3-Hexen-1-ol acetate which is emitted in high levels by the green buds has been shown to deter oviposition by *Heliothis virescens* moths on injured tobacco plants (Pichersky and Gershenzon, 2002). The midge avoids senescing flowers (Adair, 2003) for unknown reasons; however, 1-hexanol which is emitted from senescing flowers in relatively high levels is known to act as repellent to many insects (e.g. Smart and Blight, 2000; Stensmyr, 2004). It seems justified to further investigate the roles of these compounds in relation to the oviposition behaviour of the midge.

4.3. Concluding remarks and further research

This study is the first part of an investigation examining whether olfactory cues might play a role in the host finding process of *D. dielsi*. It has demonstrated that there are marked changes in the volatile compositions of different flower developmental stages in *A. cyclops*, the main host of *D. dielsi*. Electrophysiological studies to determine which compounds in each flower developmental stage elicit gall midge antennal responses are underway. Further studies investigating the scent chemistry in a phylogenetic context comparing related and unrelated *Acacia* species might explain the infrequent but successful galling by *D. dielsi* on *Acacia* species besides *A. cyclops*.

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